

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification<sup>4</sup> :C12Q 1/00, 1/26, C12M 1/34  
G01N 27/26

A1

(11) International Publication Number:

WO 89/ 08713

(43) International Publication Date:

21 September 1989 (21.09.89)

(21) International Application Number: PCT/US89/01057

(22) International Filing Date: 14 March 1989 (14.03.89)

(31) Priority Application Numbers: 168,295  
Not furnished(32) Priority Dates: 15 March 1988 (15.03.88)  
13 March 1989 (13.03.89)

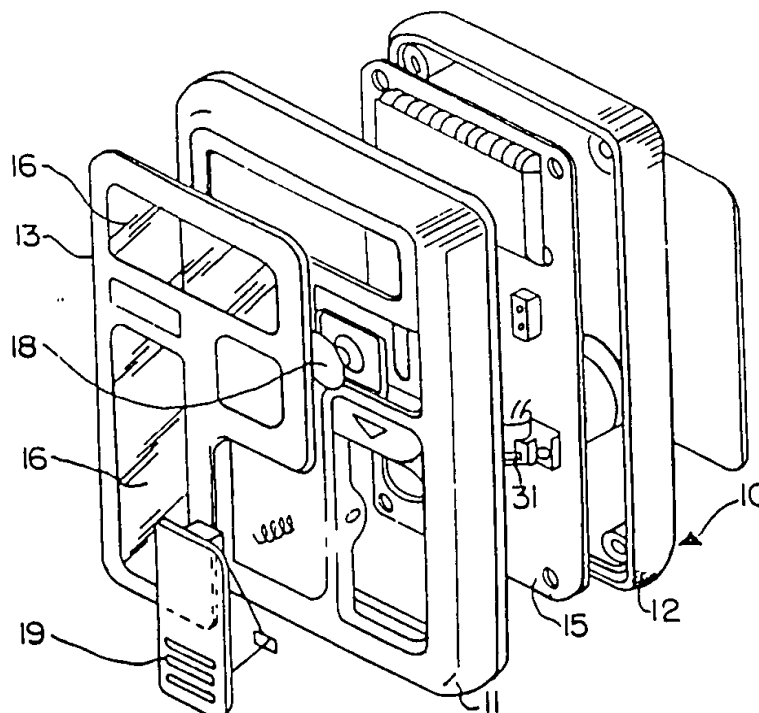
(33) Priority Country: US

(71) Applicant: LIFE-CHEK LABORATORIES [US/US]:  
4900 Perry Highway, Pittsburgh, PA 15229 (US).(72) Inventors: POTTGEN, Paul, A. ; 2335 Big Rock Road,  
Allison Park, PA 15101 (US). SZUMINSKY, Neil, J. ;  
1427 Hillsdale Avenue, Pittsburgh, PA 15216 (US).  
TALBOTT, Jonathan, L. ; 165 Woodhaven Drive,  
Mars, PA 16046 (US). JORDAN, Joseph ; 1007 Glenn  
Circle North, State College, PA 16803 (US).(74) Agent: YAHWAK, George, M.: Yahwak & Associates,  
25 Skytop Drive, Trumbull, CT 06611 (US).(81) Designated States: AT (European patent), AU, BE (Eu-  
ropean patent), CH (European patent), DE (Euro-  
pean patent), DK, FI, FR (European patent), GB  
(European patent), IT (European patent), JP, KR, LU  
(European patent), NL (European patent), NO, SE,  
SE (European patent), SU.

Published

With international search report.

(54) Title: METHOD AND APPARATUS FOR AMPEROMETRIC DIAGNOSTIC ANALYSIS



## (57) Abstract

The present invention relates to a novel method and apparatus for the amperometric determination of an analyte, and in particular, to an apparatus (10) for amperometric analysis utilizing a novel disposable electroanalytical cell (20) for the quantitative determination of biologically important compounds from body fluids.

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METHOD AND APPARATUS FOR AMPEROMETRIC  
DIAGNOSTIC ANALYSIS

The present application is a Continuation-in-Part of our  
earlier filed application, United States Serial No. 168,295,  
filed March 15, 1988.

FIELD OF THE INVENTION:

The present invention relates to a disposable  
electroanalytical cell and a method and apparatus for  
quantitatively determining the presence of biologically  
important compounds such as glucose; TSH; T4; hormones such  
as HCG; cardiac glycosides such as Digoxin; antiarrhythmics  
such as Lidocaine; antiepileptics such as phenobarbital;  
antibiotics such as Gentamicin; cholesterol; non-therapeutic  
drugs and the like from body fluids.

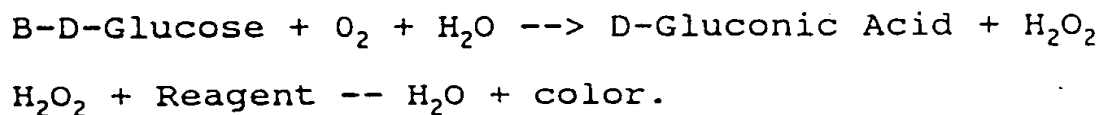
Although the present invention has broad applications,  
for purposes of illustration of the invention specific  
emphasis will be placed upon its application in  
quantitatively determining the presence of two biologically  
important compounds -- glucose and cholesterol.

WITH RESPECT TO GLUCOSE:

Diabetes, and specifically diabetes mellitus, is a  
metabolic disease characterized by deficient insulin  
production by the pancreas which results in abnormal levels  
of blood glucose. Although this disease afflicts only

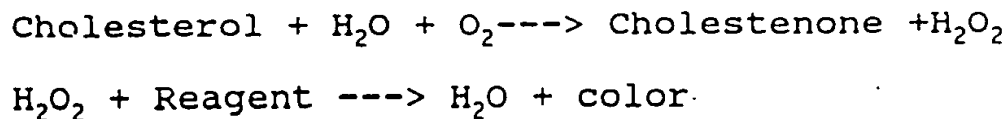
approximately 4% of the population in the United States, it is the third leading cause of death following heart disease and cancer. With proper maintenance of the patient's blood sugar through daily injections of insulin, and strict control of dietary intake, the prognosis for diabetics is excellent. However, the blood glucose levels must be closely followed in the patient either by clinical laboratory analysis or by daily analyses which the patient can conduct using relatively simple, non-technical, methods.

At the present, current technology for monitoring blood glucose is based upon visual or instrumental determination of color change produced by enzymatic reactions on a dry reagent pad on a small plastic strip. These colorimetric methods which utilize the natural oxidant of glucose to gluconic acid, specifically oxygen, are based upon the reactions:



#### WITH RESPECT TO CHOLESTEROL:

Current technology for the determination of cholesterol is also based upon similar methods. In the case of cholesterol, the methods presently used are based upon the generalized reactions:



In all present techniques, Dioxygen is the only direct oxidant used with the enzyme cholesterol oxidase for the determination of both free and total cholesterol. Using conventional test methods, oxygen must diffuse into the sensor solution during use from the surrounding air in order to provide sufficient reagent for a complete reaction with the analyte cholesterol in undiluted serum and whole blood specimens.

In both instances, the presence of the substance is determined by quantifying, either colorometrically or otherwise, the presence of hydrogen peroxide. The present methods of detection may include direct measurement of the hydrogen peroxide produced by either spectroscopic or electrochemical means and indirect methods in which the hydrogen peroxide is reacted with various dyes, in the presence of the enzyme peroxidase, to produce a color that is monitored.

While relatively easy to use, these tests require consistent user technique in order to yield reproducible results. For example, these tests require the removal of blood from a reagent pad at specified and critical time intervals. After the time interval, excess blood must be removed by washing and blotting, or by blotting alone, since the color measurement is taken at the top surface of the reagent pad. Color development is either read immediately or after a specified time interval.

These steps are dependent upon good and consistent operating technique requiring strict attention to timing. Moreover, even utilizing good operating technique, colorimetric methods for determining glucose, for example, have been shown to have poor precision and accuracy, particularly in the hypoglycemic range. Furthermore, instruments used for the quantitative colorimetric measurement vary widely in their calibration methods: some provide no user calibration while others provide secondary standards.

Because of the general lack of precision and standardization of the various methods and apparatus presently available to test for biologically important compounds in body fluids, some physicians are hesitant to use such equipment for monitoring levels or dosage. They are particularly hesitant in recommending such methods for use by the patients themselves. Accordingly, it is desirable to have a method and apparatus which will permit not only physician but patient self-testing of such compounds with greater reliability.

The present invention addresses the concerns of the physician by providing enzymatic amperometry methods and apparatus for monitoring compounds within whole blood, serum, and other body fluids. Enzymatic amperometry provides several advantages for controlling or eliminating operator dependant techniques as well as providing a greater

linear dynamic range. A system based on this type of method could address the concerns of the physician hesitant to recommend self-testing for his patients.

Enzymatic amperometry methods have been applied to the laboratory based measurement of a number of analytes including glucose, blood urea nitrogen, and lactate. Traditionally the electrodes in these systems consist of bulk metal wires, cylinders or disks imbedded in an insulating material. The fabrication process results in individualistic characteristics for each electrode necessitating calibration of each sensor. These electrodes are also too costly for disposable use, necessitating meticulous attention to electrode maintenance for continued reliable use. This maintenance is not likely to be performed properly by untrained personnel (such as patients), therefore to be successful, an enzyme amperometry method intended for self-testing (or non-traditional site testing) must be based on a disposable sensor that can be produced in a manner that allows it to give reproducible output from sensor to sensor and at a cost well below that of traditional electrodes.

The present invention address these requirements by providing miniaturized disposable electroanalytic sample cells for precise micro-aliquote sampling, a self-contained, automatic means for measuring the electrochemical reduction

of the sample, and a method for using the cell and apparatus according to the present invention.

The disposable cells according to the present invention are preferably laminated layers of metallized plastic and nonconducting material. The metallized layers provide the working and reference electrodes, the areas of which are reproducibly defined by the lamination process. An opening through these layers is designed to provide the sample-containing area or cell for the precise measurement of the sample. The insertion of the cell into the apparatus according to the present invention, automatically initiates the measurement cycle.

To better understand the process of measurement, a presently preferred embodiment of the invention is described which involves a two-step reaction sequence utilizing a chemical oxidation step using other oxidants than oxygen, and an electro-chemical reduction step suitable for quantifying the reaction product of the first step. One advantage to utilizing an oxidant other than dioxygen for the direct determination of an analyte is that they may be prepositioned in the sensor in a large excess of the analyte and thus ensure that the oxidant is not the limiting reagent (with dioxygen, there is normally insufficient oxidant initially present in the sensor solution for a quantitative conversion of the analyte).



In the oxidation reaction, a sample containing glucose, for example, is converted to gluconic acid and a reduction product of the oxidant. This chemical oxidation reaction has been found to precede to completion in the presence of an enzyme, glucose oxidase, which is highly specific for the substrate B-D-glucose, and catalyzes oxidations with single and double electron acceptors. It has been found, however, that the oxidation process does not proceed beyond the formation of gluconic acid, thus making this reaction particularly suited for the electrochemical measurement of glucose.

In a presently preferred embodiment, oxidations with one electron acceptor using ferricyanide, ferricinium, cobalt (III) orthophenantroline, and cobalt (III) dipyriddy are preferred. Benzoquinone is a two electron acceptor which also provides excellent electro-oxidation characteristics for amperometric quantitation.

Amperometric determination of glucose, for example, in accordance with the present invention utilizes Cottrell current micro-chronoamperometry in which glucose plus an oxidized electron acceptor produces gluconic acid and a reduced acceptor. This determination involves a preceding chemical oxidation step catalyzed by a bi-substrate bi-product enzymatic mechanism as will become apparent throughout this specification.

In this method of quantification, the measurement of a diffusion controlled current at an accurately specified time (e.g. 20, 30, or 50 seconds, for example) after the instant of application of a potential has the applicable equation for amperometry at a controlled potential ( $E = \text{constant}$ ) of:

$$i_{\text{COTTRELL}} = nFA( D t )^{-0.5} C_{\text{METABOLITE}}$$

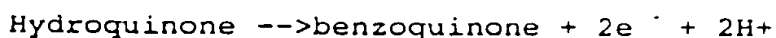
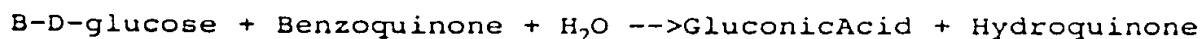
at  $t > 0$                       at  $t = 0$

where  $i$  denotes current,  $nF$  is the number of coulombs per mole,  $D$  is the diffusion coefficient of the reduced form of the reagent,  $t$  is the preset time at which the current is measured, and  $C$  is the concentration of the metabolite. Measurements by the method according to the present invention of the current due to the reoxidation of the acceptors were found to be proportional to the glucose concentration in the sample.

The method and apparatus of the present invention permit, in preferred embodiments, direct measurements of blood glucose, cholesterol and the like. Furthermore, the sample cell according to the present invention, provides the testing of controlled volumes of blood without premeasuring. Insertion of the sampling cell into the apparatus thus permits automatic functioning and timing of the reaction allowing for patient self-testing with a very high degree of precision and accuracy.

One of many of the presently preferred embodiments of the invention for use in measuring B-D glucose is described in detail to better understand the nature and scope of the invention. In particular, the method and apparatus according to this embodiment are designed to provide clinical self-monitoring of blood glucose levels by a diabetic patient. The sample cell of the invention is used to control the sampling volume and reaction media and acts as the electrochemical sensor. In this described embodiment, benzoquinone is used as the electron acceptor.

The basic chemical binary reaction utilized by the method according to the present invention is:



The first reaction is an oxidation reaction which proceeds to completion in the presence of the enzyme glucose oxidase. Electrochemical oxidation takes place in the second part of the reaction and provides the means for quantifying the amount of hydroquinone produced in the oxidation reaction. This holds true whether catalytic oxidation is conducted with two-electron acceptors or one electron acceptors such as ferricyanide [wherein the redox couple would be  $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ ], ferricinium, cobalt III tris orthophenantroline and cobalt (III) trisdipyridyl.

Catalytic oxidation by glucose oxidase is highly specific for B-D-glucose, but is nonselective as to the

oxidant. It has now been discovered that the preferred oxidants described above have sufficiently positive potentials to convert substantially all of the B-D-glucose to gluconic acid. Furthermore, this system provides a means  
5 by which amounts as small as 1 mg of glucose (in the preferred embodiment) to 1000 mg of glucose can be measured per deciliter of sample - results which have not previously been obtained using other glucose self-testing systems.

The sensors containing the chemistry to perform the  
10 desired determination, constructed in accordance with the present invention, are used with a portable meter for self-testing systems. In use the sensor is inserted into the meter which turns the meter on and initiates a wait for the application of the sample. The meter recognizes sample  
15 application by the sudden charging current flow that occurs when the electrodes and the overlaying reagent layer are initially wetted by the sample fluid. Once the sample application is detected, the meter begins the reaction incubation step (the length of which is chemistry dependent)  
20 to allow the enzymatic reaction to reach completion. This period is on the order of 15 to 90 seconds for glucose, with incubation times of 20 to 45 seconds preferred. Following the incubation period, the instrument then imposes a known potential across the electrodes and measures the current at  
25 specific time points during the Cottrell current decay. Current measurements can be made in the range of 2 to 30

seconds following potential application with measurement times of 10 to 20 seconds preferred. These current values are then used to calculate the analyte concentration which is then displayed. The meter will then wait for either the user to remove the sensor or for a predetermined period before shutting itself down.

The present invention provides for a measurement system that eliminates several of the critical operator dependant variables that adversely affect the accuracy and reliability and provides for a greater dynamic range than other self-testing systems.

These and other advantages of the present invention will become apparent from a perusal of the following detailed description of one embodiment presently preferred for measuring glucose and another for measuring cholesterol which is to be taken in conjunction with the accompanying drawings in which like numerals indicate like components and in which:

FIG. 1 is an exploded view of a portable testing apparatus according to the present invention;

FIG. 2 is a plan view of the sampling cell of the present invention;

FIG. 3 is an exploded view of the sample cell shown in Figure 2;

FIG. 4 is an exploded view of another embodiment of a sample cell according to the invention;

FIG. 5 is a plan view of the cell shown in Figure 4;

FIG. 6 is still another embodiment of a sample cell;

FIG. 7 is a graph showing current as a function of glucose concentration;

5 FIG. 8 is a graphical presentation of Cottrell current as a function of glucose concentration; and

FIG. 9 is a presently preferred block diagram of an electrical circuit for use in the apparatus shown in Figure 1.

10 FIG. 10 is a preferred embodiment of the electrochemical cell.

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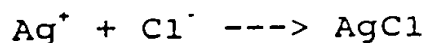
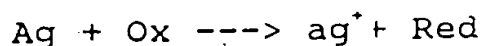
With specific reference to Figure 1, a portable electrochemical testing apparatus 10 is shown for use in patient self-testing, such as, for example, for blood glucose levels. Apparatus 10 comprises a front and back housing 11 and 12, respectively, a front panel 13 and a circuit board 15. Front panel 13 includes graphic display panels 16 for providing information and instructions to the patient, and direct read-out of the test results. While a start button 18 is provided to initiate an analysis, it is

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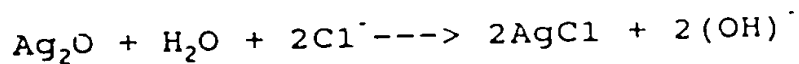
preferred that the system begin operation when a sample cell 20 is inserted into the window 19 of the apparatus.

With reference to Figure 3, sample cell 20 is a metallized plastic substrate having a specifically-sized opening 21 which defines a volumetric well 21, when the cell is assembled, for containing a reagent pad and the blood to be analyzed. Cell 20 comprises a first 22 and second 23 substrate which may be preferable made from styrene or other substantially non-conducting plastic. Positioned on second substrate 23 is reference electrode 24. Reference electrode 24 may be preferably manufactured, for example, by vapor depositing the electrode onto a substrate made from a material such as the polyimide Kapton. In the preferred embodiment, reference electrode 24 is a silver-silver chloride electrode. This electrode can be produced by first depositing a silver layer silver chloride by either chemical or electrochemical means before the substrate is used to construct the cells. The silver chloride layer may even be generated in-situ on a silver electrode when the reagent layer contains certain of the oxidants, such as ferricyanide, and chloride as shown in the following reactions:



Alternatively the silver-silver chloride electrode can be produced by depositing a layer of silver oxide (by reactive

sputtering) onto the silver film. This silver oxide layer is then converted in-situ at the time of testing to silver chloride according to the reaction:



5 when the sensor is wetted by the sample fluid and reconstitutes the chloride containing reagent layer. The silver electrode with a layer containing silver chloride.

The reference electrode may also be of the type generally known as a "pseudo" reference electrode which  
10 relies upon the large excess of the oxidizing species to establish a known potential at a noble metal electrode. In a preferred embodiment, two electrodes of the same noble metal are used, however one is generally of greater surface area and is used as the reference electrode. The large  
15 excess of the oxidized species and the larger surface area of the reference resists a shift of the potential of the reference electrode.

Indicator or working electrode 26 can be either a strip of platinum, gold, palladium or metallized plastic  
20 positioned on reference electrode 24 or alternately the working electrode 26 and the reference electrode may be manufactured as a coplanar unit with electrode 26 being sandwiched between coplanar electrode 24 material. Preferable, sample cell 20 is prepared by sandwiching or  
25 laminating the electrodes between the substrate to form a composite unit.



As shown in Figure 2, first substrate 22 is of a slightly shorter length so as to expose and end portion 27 of electrodes 24 and 26 and allow for electrical contact with the testing circuit contained in the apparatus. In this embodiment, after a sample has been positioned within well 21, cell 20 is pushed into window 19 of the front panel to initiate testing. In this embodiment, a reagent may be applied to well 21, or, preferably, a pad of dry reagent is positioned therein and a sample (drop) of blood is placed into the well 21 containing the reagent.

Referring to Figures 4-6, alternative embodiments of sample cell 20 are shown. In Figure 4, sample cell 120 is shown having first 122 and second 123 substrates. Reference electrode 124 and working electrode 126 are laminated between substrates 122 and 123. Opening 121 is dimensioned to contain the sample for testing. End 130 is designed to be inserted into the apparatus, and electrical contact is made with the respective electrodes through cut-outs 131 and 132 on the cell. Reference electrode 124 also includes cut out 133 to permit electrical contact with working electrode 126.

In Figure 6, working electrode 226 is folded, thereby providing increased surface area around opening 221, to achieve increased sensitivity or specificity. In this case, reference electrode 224 is positioned beneath working electrode 226. Working electrode includes cut out 234 to

permit electrical contact with reference electrode 224 through cut out 231 in substrate 222. End 230 of substrate 222 also includes cut out 232 to permit electrical contact with working electrode 226.

5 The sample cell according to the present invention is positioned through window 19 to initiate the testing procedure. Once inserted, a potential is applied at portion 27 of the sample cell across electrodes 24 and 26 to detect the presence of the sample. Once the sample's presence is  
10 detected, the potential is removed and the incubation period initiated. Optionally during this period, a vibrator means 31 may be activated to provide agitation of the reagents in order to enhance dissolution (an incubation period of 20 to 45 seconds is conveniently used for the determination of  
15 glucose and no vibration is normally required). An electrical potential is next applied at portion 27 of the sample cell to electrodes 24 and 26 and the current through the sample is measured and displayed on display 16.

To fully take advantage of the above apparatus, the  
20 needed chemistry for the self testing systems is incorporated into a dry reagent layer that is positioned onto the disposable cell creating a complete sensor for the intended analyte. The disposable electrochemical cell is constructed by the lamination of metallized plastics and  
25 nonconducting materials in such a way that there is a precisely defined working electrode area. The reagent layer

is either directly coated onto the cell or preferably incorporated (coated) into a supporting matrix such as filter paper, membrane filter, woven fabric or non-woven fabric, which is then placed into the cell. When a supporting matrix is used, its pore size and void volume can be adjusted to provide the desired precision and mechanical support. In general, membrane filters or nonwoven fabrics provide the best materials for the reagent layer support. Pore sizes of 0.45 to 50um and void volumes of 50-90% are appropriate. The coating formulation generally includes a binder such as gelatin, carrageenan, methylcellulose, polyvinyl alcohol, polyvinylpyrrolidone, etc., that acts to delay the dissolution of the reagents until the reagent layer has adsorbed most of the fluid from the sample. The concentration of the binder is generally on the order of 0.1 to 10% with 1-4% preferred.

The reagent layer imbibes a fixed amount of the sample fluid when it is applied to the surface of the layer thus eliminating any need for premeasurement of sample volume. Furthermore, by virtue of measuring current flow rather than reflected light, there is no need to remove the blood from the surface of the reagent layer prior to measurement as there is with reflectance spectroscopy systems. While the fluid sample could be applied directly to the surface of the reagent layer, to facilitate spread of blood across the entire surface of the reagent layer the sensor preferably

includes a dispersing spreading or wicking layer. This layer, generally a non-woven fabric or adsorbant paper, is positioned over the reagent layer and acts to rapidly distribute the blood over the reagent layer. In some applications this dispersing layer could incorporate additional reagents.

For glucose determination, cells utilizing the coplanar design were constructed having the reagent layer containing the following formulations:

10	Glucose oxidase	600u/ml
	Potassium Ferricyanide	0.4M
	Phosphate Buffer	0.1M
	Potassium Chloride	0.5M
	Gelatin	2.0g/dl

15 This was produced by coating a membrane filter with a solution of the above composition and air drying. The reagent layer was then cut into strips that just fit the window opening of the cells and these strips were placed over the electrodes exposed within the windows. A wicking layer of a non-woven rayon fabric was then placed over this reagent layer and held in place with an overlay tape.

In order to prove the application of the technology according to the present invention, a large number of examples were run in aqueous solution at 25°C. The electrolyte consisted of a phosphate buffer of pH 6.8 which was about 0.1 molar total phosphate and 0.5 M potassium

chloride reagent. The potentials are referenced to a normal hydrogen electrode (NHE). In these tests it was found that any potential between approximately +0.8 and 1.2 volt (vs NHE) is suitable for the quantification of hydroquinone when benzoquinone is used as the oxidant. The limiting currents are proportional to hydroquinone concentrations in the range between 0.0001 M and 0.050 M.

Determination of glucose by Cottrell current ( $i_t$ ) microchronoamperometry with the present method is created in the reaction of hydroquinone to benzoquinone. Cottrell currents decay with time in accordance with the equation:

$$i_t \cdot t^{1/2} = \text{const}$$

The main difference between these two techniques consists of applying the appropriate controlled potential after the glucose-benzoquinone reaction is complete and correlating glucose concentrations with Cottrell currents measured at a fixed time thereafter. The current-time readout is shown in Figure 8. Proportionality between glucose concentrations and Cottrell currents (recorded at  $t = 30$  seconds after the application of potential) is shown in Figure 7.

It should be noted that Cottrell chronoamperometry of metabolites needs the dual safeguards of enzymatic catalysis and controlled potential electrolysis. Gluconic acid yields of 99.9+ percent were attained in the presence of glucose oxidase. Concomitantly, equivalent amounts of benzoquinone

were reduced to hydroquinone, which was conveniently quantitated in quiescent solutions, at stationary palladium thin film anodes or sample cells.

The results of these many tests demonstrates the microchronoamperometric methodology of the present invention and its practical for glucose self-monitoring by diabetics.

In a presently preferred embodiment of the invention utilizing ferrocyanide, a number of tests were run showing certain improved operating capabilities.

Referring to Figure 9, a schematic diagram of a preferred circuit 15 for use in the apparatus 10 is shown. Circuit 15 includes a microprocessor and LCD panel 16. The working and reference electrodes on the sample cell 20 make contact at contacts W (working electrode) and R (reference electrode), respectively. Voltage reference 41 is connected to battery 42 through analogue power switch 43. Current from the electrodes W and R is converted by adjustable resistor 44, and voltage to frequency converter 46 electrically connected to the microprocessor. Other circuits within the skills of a practiced engineer can obviously be utilized to obtain the advantages of the present invention.

With regard to Figure 10, cell 400 consists of coplanar working 426 and reference 424 electrodes laminated between an upper 422 and lower 426 nonconducting material. Lamination is on an adhesive layer 425. The upper material

422 includes a die cut opening 428 which, along with the width of the working electrode material defines the working electrode area and provides (with an overlapping reagent layer not depicted) the sampling port of the cell. At one  
5 end of cell 400 is an open area 427 similar to end position 27.

The efficiency of using the apparatus according to the present invention to provide a means for in-home self testing by patients such as diabetics (in the preferred  
10 embodiment) can be seen in the following table in which the technology according to the present invention is compared to four commercially available units. As will be seen, the present invention is simpler, and in this instance simplicity breeds consistency in results.

GLUCOSE SYSTEM COMPARISONS

<u>Steps</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>Present Inven- tion</u>
Turn Instrument On	X	X	X	X	X
Calibrate Instrument	X	X			
Finger Puncture	X	X	X	X	X
Apply Blood	X	X	X	X	X
Initiate Timing Sequence	X	X	X		
Blot	X	X	X		
Insert Strip to Read	X	X	X	X	
Read Results	<u>X</u>	<u>X</u>	<u>X</u>	<u>X</u>	<u>X</u>
Total Steps Per Testing	8	8	7	5	4
Detection System	RS*	RS	RS	RS	Polaro- graphic
Range (mg/dl)	10-400	40-400	25-450	40-400	0-1000
CV** Hypoglycemic	15%	15%			5%
Euglycemic	10%	10%			3%
Hyperglycemic	5%	5%			2%
Correlation	0.921	0.862			0.95

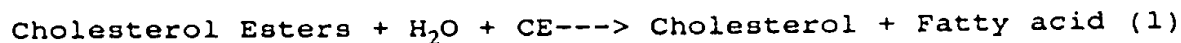
(\*RS - Reflectance Spectroscopy)

\*\*Coefficient of variation



5 With specific regard to the determination of cholesterol utilizing the present invention, the generalized chemistry may be depicted as:

## Scheme I

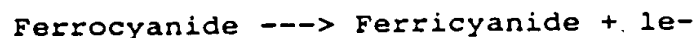
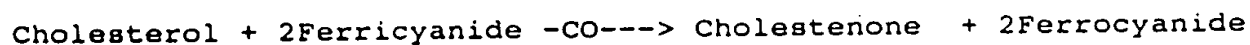


where the enzymes cholesterol esterase (CE) and cholesterol oxidase (CO) catalyze reactions 1 and 2 respectively and CO permits electron transfer with a variety of electroactive couples (Ox and Red). Reaction 2 is novel in that electron acceptors other than dioxygen may be used to oxidize cholesterol in the presence of the enzyme cholesterol oxidase. Reaction 1 is well known to those in the field and is necessary for the determination of total cholesterol (free cholesterol and cholesterol esters). Reaction 3 is an electro-oxidation process for probing and quantitating the cholesterol.

Utilizing alternative oxidants according to the present invention, the specific reactions become:

25 A:

Reaction 1 above



B:

## Reaction 1 above

Cholesterol + Benzoquinone -CO---> Cholestenone + Hydroquinone

Hydroquinone ---> Benzoquinone + 2H<sup>+</sup> + 2e<sup>-</sup>

5 Cholesterol oxidase (CO) from a variety of sources will catalyze electron transfer from cholesterol to a variety of the oxidants including benzoquinone, benzoquinone derivatives such as methylbenzoquinone, ethylbenzoquinone, chlorobenzoquinone, ortho-benzoquinone (oxidized form of catechol), benzoquinonesulfonate, and potassium ferricyanide. It is also anticipated that the enzyme will allow electron transfer with other alternate oxidants. As indicated in Reaction 3, the reduced product can then be monitored amperometrically for the quantitative  
10 determination of cholesterol.  
15

Sources of the enzyme catalyzing the oxidation of cholesterol with alternate oxidants include CO from Nocardia, Streptomyces, Schizophyllum, Pseudomonas, and Brevibacterium; experimental conditions under which it is able to rapidly catalyze the oxidation of cholesterol by benzoquinone or any of the other oxidants depend somewhat upon the source of the enzyme. For example, CO from Streptomyces rapidly catalyzes substrate oxidation with  
20 benzoquinone in phosphate buffer in the presence of any of a variety of the surfactants including octylgluconopyranoside and CHAPSO; the same reaction under  
25

identical conditions with CO from either *Brevibacterium* or *Nocardia* is slower. However, both *Nocardia* and *Brevibacterium* sources are active catalysts for cholesterol oxidation by alternate oxidants under other conditions.

The oxidant also plays a role in which the enzyme is most active. For example, cholesterol oxidase from *Nocardia* rapidly catalyzes substrate oxidation with benzoquinone in 0.2 molar TRIS buffer and 3 g/dL CHAPSO but is slower with ferricyanide under identical conditions; the *Brevibacterium* source of the enzyme is relatively inactive with ferricyanide in TRIS buffer with a variety of surfactants but when benzoquinone is used as the oxidant the reaction is very fast. Alternatively, the *Schizophyllum* source of the enzyme CO rapidly catalyzes the oxidation of cholesterol in phosphate buffer with either ferricyanide or benzoquinone and with a variety of surfactants as activators.

As indicated, cholesterol oxidase will catalyze the oxidation of cholesterol by ferricyanide. Additional examples where CO catalyzes cholesterol oxidation by ferricyanide include a *Nocardia* source in TRIS buffer with a variety of surfactants including sodium deoxycholate, sodium taurodeoxycholate, CHAPS, Thesit, and CHAPSO. Furthermore, CO from *Nocardia* will also catalyze substrate oxidation with ferricyanide in phosphate buffer with sodium dioctylsulfosuccinate, sodium deoxycholate, sodium taurodeoxycholate, and Triton X-100. The buffer

concentration is from 0.1 to 0.4 molar. Surfactant concentration for maximum activity of the oxidase enzyme varies with each detergent. For example, with deoxycholate or taurodeoxycholate, the enzyme in 0.2 M TRIS is most active with detergent in the range from 20 to 90 millimolar. However, enzyme catalytic activity is observed up to and through a 10% concentration. With octyl-gluconopyranoside, the maximum activity of the enzyme with the oxidant ferricyanide occurs at a detergent concentration of approximately 1.2%; however, the enzyme still maintains activity at higher and lower concentrations of the surfactant.

Both esterase and CO require a surfactant for high activity. Specific surfactants include sodium deoxycholate, sodium taurodeoxycholate, sodium glycodeoxycholate, CHAPS (3-(3-chloroamidopropyl)dimethylammonio-1-propanesulfonate), CHAPSO (3-(3-chloroamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate), octyl-gluconopyranoside, octyl-thiogluconopyranoside, nonyl-gluconopyranoside, dodecyl-gluconopyranoside, Triton X-100, Dioctyl sulfosuccinate, Thesit (Hydroxypolyethoxydodecane), and lecithin (phosphatidylcholine). Buffers acceptable for this reaction to occur with the enzyme include phosphate, TRIS, MOPS, MES, HEPES, Tricine, Bicine, ACES, CAPS, and TAPS. An alternate generalization reaction scheme for the measurement of cholesterol in serum and other biological fluids is given

## Scheme II

Cholesterol Esters -CE---> Cholesterol + Fatty Acid 1

Cholesterol + Ox<sub>1</sub> -CO---> Cholestenone + Red<sub>1</sub> 4

Red<sub>1</sub> + Ox<sub>2</sub> ---> Ox<sub>1</sub> + Red<sub>2</sub> 5

5 Red<sub>2</sub> ----> Ox<sub>2</sub> + e- 6

where Ox<sub>1</sub> and Red<sub>2</sub> function as an electron mediator couple between the cholesterol and the electroactive couple Ox<sub>2</sub>/Red<sub>2</sub>. In this case, Ox<sub>1</sub> and Red<sub>1</sub> need not be electroactive because they do not have to participate in the electrooxidation process (Reaction 6). However, from both a thermodynamic and kinetic perspective, this couple with the assistance of the enzyme cholesterol oxidase must be able to accept electrons from cholesterol and relay them to the electroactive couple (Ox<sub>2</sub>/Red<sub>2</sub>).

15 Specific examples of this chemistry include

## Example 1

## Reaction 1 above

Cholesterol + Benzoquinone -CO---> Cholestenone + Hydroquinone

Hydroquinone + 2Ferricyanide ----> Benzoquinone + 2Ferrocyanide

20 Ferrocyanide ----> Ferricyanide + 1e-

Scheme II is beneficial when the rate of reaction of cholesterol with the electroactive oxidant as in Scheme I is so slow that it precludes its use in a practical sensor. As mentioned above, Scheme II is also beneficial when the

electron mediator itself ( $Ox_1/Red_1$ ) is either not electroactive or exhibits poor electrochemistry under conditions of the enzyme chemistry. It is under these conditions that Scheme II is particularly applicable. Other  
5 electron mediators ( $Ox_1/Red_1$ ) between cholesterol and ferricyanide for use in Scheme II may be possible including phenazine ethosulfate, phenazine methosulfate, tetramethylbenzidine, derivatives of benzoquinone, naphthoquinone and naphthoquinone derivatives, anthraquinone  
10 and anthraquinone derivatives, catechol, phenylenediamine, tetramethylphenenediamine, and other derivatives of phenylenediamine.

Furthermore, while it is understood that the oxidized form of the electron relay accepts electrons from  
15 cholesterol, in the sensor either the oxidized or the reduced form of the mediator may be incorporated provided it reacts rapidly with both cholesterol and ferricyanide. If the reduced form is sufficiently stable and the oxidized form is not, then reductant, may be incorporated into the  
20 sensor in relatively small quantity (in comparison with the analyte to be determined) and still provide the electron relay. However, this causes a corresponding background signal that must be accounted for. The reductant, must also be isolated from ferricyanide in the sensor by incorporation  
25 into a separate reagent layer.

Several formulations of the above chemistries encompassing both Schemes I and II have been prepared as dry films on membranes. These membranes are positioned in the sensor which can then be used for the determination of cholesterol. A preferred formulation of the reagents involving Scheme II consists of the following

Cholesterol Esterase @ 400 Units/mL

Cholesterol Oxidase from Streptomyces @ 200 Units/mL

0.05 molar Potassium Ferricyanide

0.5 molar Potassium Chloride

0.2 molar Phosphate, pH 6.9

3 g/dL CHAPSO

2 g/dL gelatin

and 0.0001 molar hydroquinone (in the spreading or wicking layer).

The concentrations provided are that of the solutions which are coated onto porous supports, filter paper or membranes; these concentrations are reestablished when the membrane imbibes the serum or whole blood specimen. For cholesterol determinations larger pore sizes in the filter support are necessary than that used for glucose. This is because the cholesterol resides in the serum in large lipoproteins (chylomicrons, LDL, VLDL, and HDL) which must penetrate the various layers of the sensor until they reach the reagents. The surfactants to a major extent break these natural micelles up into smaller micelles providing a greater total

surface area on which the enzymes catalyze the reaction. Due to the instability of benzoquinone a small quantity of hydroquinone, which is more stable by nature of its lower vapor pressure, is incorporated into the sensor to assist electron mediation between cholesterol and ferricyanide. Upon introduction of the serum specimen into the sensor the hydroquinone is oxidized to benzoquinone; the benzoquinone is then free to pick up electrons from the substrate and cycle them to ferricyanide. Under these conditions the rate of the reaction of cholesterol with a small quantity of benzoquinone is more rapid than that with a large excess of ferricyanide.

An alternate and preferred formulation of reagents utilizing Scheme II that may be incorporated into the reagent layer of the sensor is:

Cholesterol Oxidase from Streptomyces @ 200 Units/mL

Lipase from Candida @ 500 Units/mL

3 g/dL CHAPSO

0.2 molar TRIS, pH 7.5

0.05 molar Potassium Ferricyanide

0.5 molar Potassium Chloride

0.05 molar  $MgCl_2$

2 g/dL gelatin

and 0.001 molar hydroquinone (in the spreading layer).

The magnesium salt in this formulation increases stability of the esterase enzyme in the phosphate-free reagent layer;



Lipase assists the break up of the lipoproteins. With these dry reagent layers incorporated into the sensor and using the evaluation methodology as described, the following results were obtained.

<u>Serum Cholesterol, mg%</u>	<u>Average Current, uA</u>
91	19.3
182	27.2
309	38.5

These results demonstrate the quantitative response of the sensor to serum cholesterol levels.

Alternate and preferred embodiment of the sensor utilizing Scheme I is provided by reagent compositions:

Cholesterol Esterase @ 400 Units/mL

Cholesterol Oxidase from Nocardia @ 200 Units/mL

1 g/dL Triton X-100

0.1 molar TRIS buffer, pH 8.6

0.2 molar Potassium Ferricyanide

0.5 molar Potassium Chloride

0.02 molar  $MgCl_2$

2 g/dL gelatin

OR

Cholesterol Esterase @ 200 Units/mL

Cholesterol Oxidase from Streptomyces @ 200 Units/mL

0.06 molar Sodium deoxycholate

0.1 molar TRIS buffer, pH 8.6

0.2 molar Potassium Ferricyanide

0.5 molar Potassium Chloride

2 g/dL gelatin

Thus, while we have illustrated and described the preferred embodiment of my invention, it is to be understood  
5 that this invention is capable of variation and modification, and we therefore do not wish or intend to be limited to the precise terms set forth, but desire and intend to avail ourselves of such changes and alterations which may be made for adapting the invention of the present  
10 invention to various usages and conditions. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents, and therefore within the purview, of the following claims. The terms and expressions which have been employed in the foregoing  
15 specifications are used therein as terms of description and not of limitation, and thus there is no intention, in the use of such terms and expressions, of excluding equivalents of the features shown and described or portions thereof, it being recognized that the scope of the invention is defined  
20 and limited only by the claims which follow.

Having thus described our invention and the manner and process of making and using it in such full, clear, concise, and exact terms so as to enable any person skilled in the

art to which it pertains, or to with which it is most nearly connected, to make and use the same.

WE CLAIM

1. A method for measuring the amount of a selected compound in body fluids comprising

- 5           a. placing a sample of fluid to be tested in a sample cell having first and second electrodes;
- b. mixing said sample with an oxidant and a buffer;
- c. applying a potential across said electrodes  
10           and sample; and
- d. measuring the resultant current to determine the concentration of said select compound present in said sample.

15           2. A method as set forth in Claim 1, wherein the compound is selected from the group consisting of glucose, cholesterol, TSH, T4, hormones, antiarrhythmics, antiepileptics and nontherapeutic drugs.

20           3. A method as set forth in Claim 1, wherein the oxidant is selected from the group consisting of benzoquinone, ferricyanide, ferricinium, cobalt (III), tris orthophenantroline, and cobalt (III) tridipyridyl.

4. A method as set forth in Claim 1 wherein the electrodes of the sample cell comprise a reference and a working electrode.

5. A sample cell comprising

a. first and second nonconductive substrates, said first substrate having an opening therethrough;

b. a metallized first electrode positioned on one of said second substrate; and

c. a second electrode positioned on said first electrode, whereby said first electrode is positioned over a portion of said second electrode to form a laminate with said first and second electrodes positioned therebetween and said opening exposing said electrodes to define a sample well.

6. A sample cell as set forth in Claim 5, wherein first electrode comprises a reference electrode and said second electrode is a working electrode.

7. A sample cell comprising first and second nonconducting substrates and first and second metallized electrodes, said first electrode being positioned on said second substrate and said second electrode including a

nonconducting substrate interpositioned between said first and second substrates; an opening through said first substrate and said second electrode, said opening defining a sample well.

5

8. A sample cell as set forth in Claim 7, wherein said first electrode is a working electrode and said second electrode is a reference electrode and wherein said opening in the second electrode is smaller than said opening of the first substrate.

10

9. A sample cell as set forth in Claim 7, wherein said first electrode is a reference electrode and said second electrode is a working electrode.

15

10. A sample cell comprising first and second nonconducting substrates and a working end reference electrode, said reference electrode comprising a metallized layer on said second substrate and said working electrode comprising a metallized layer on a nonconducting support laminated between said first and second substrates, said working electrode including a convoluted portion with an opening therethrough defining a sample well, and an opening

20

through said first substrate aligned with said opening in said working electrode.

5 11. A laminated sample cell as set forth in Claim 10, wherein said first substrate includes a pair of notches exposing and defining, a contact area on said working electrode and a contact area on said reference electrode and a notch in said working electrode positioned under said notch in said first substrate to define said reference  
10 electrode contact area.

12. An apparatus for measuring blood glucose comprising

- a. a housing;
- b. circuit board mounted within said housing  
15 having thereon electrical contacts adapted to electrically contact the electrodes of a sample cell containing a sample to be measures;
- c. a sample cell containing first and second electrodes and a well for containing a sample to be  
20 tested;
- d. an opening in said housing to permit a sample cell to be inserted into said housing and contact said electrical contact;

e. means for applying an electrical potential to said electrodes; and

f. means for measuring the electrical current through said sample.

5

13. Apparatus set forth in Claim 12 wherein said means for measuring electrical current through said sample includes a microprocessor.

10 14. Apparatus as set forth in Claim 12 wherein said window includes means for initiating the vibration of sample cell and electrical potential upon insertion of said sample cell.

15 15. Apparatus as set forth in Claim 12 wherein said electrodes of said sample cell comprise a reference and working electrode.



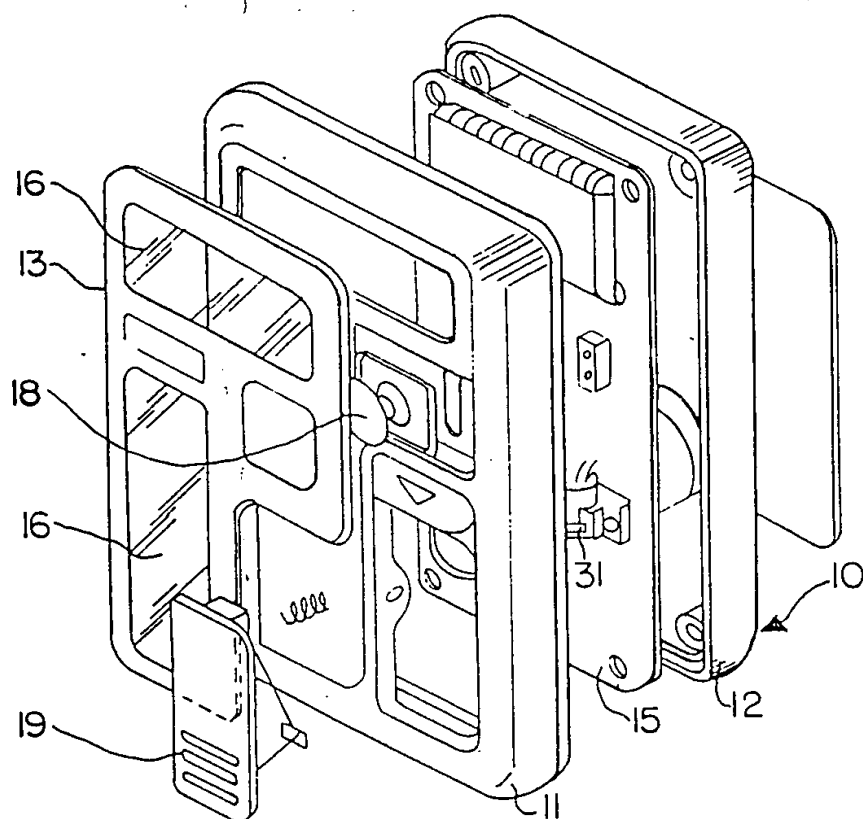


FIG. 1

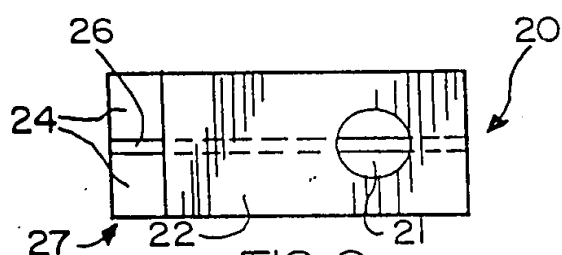


FIG. 2

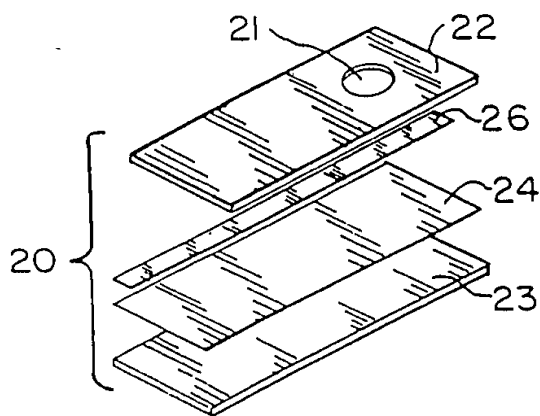
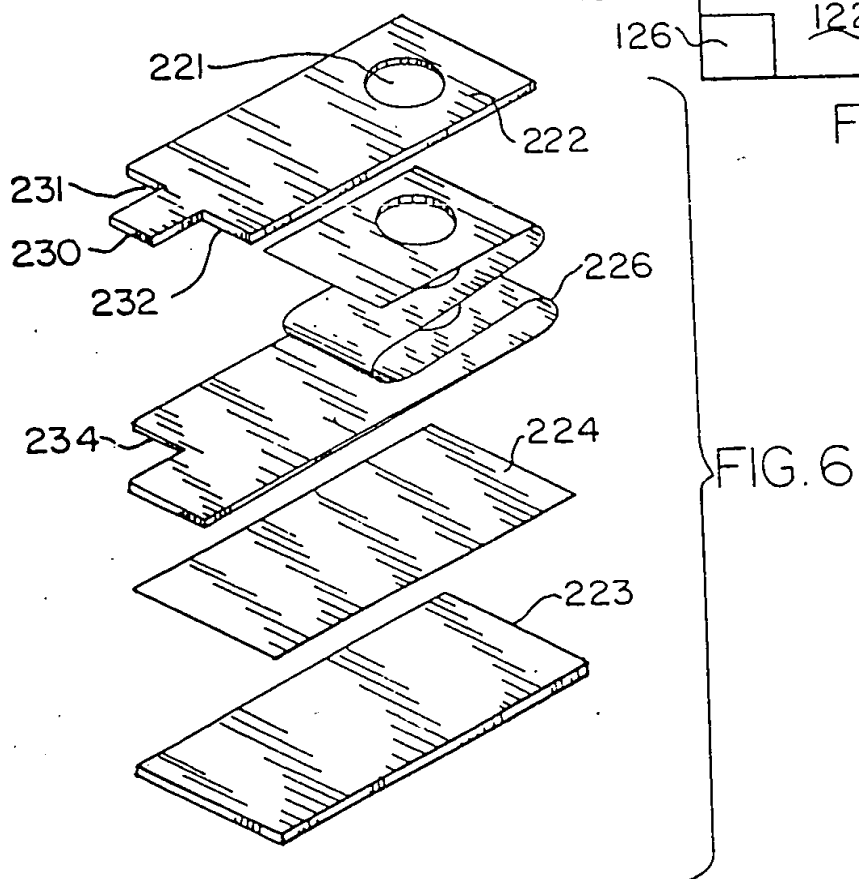
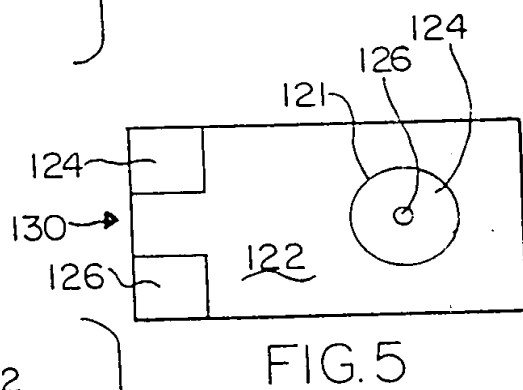
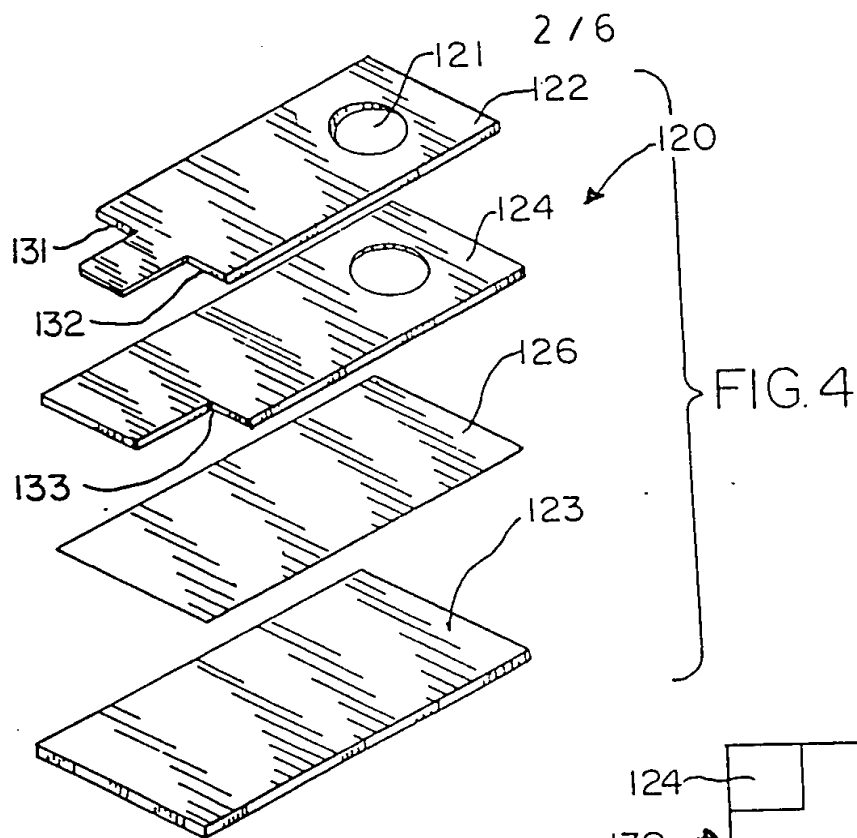
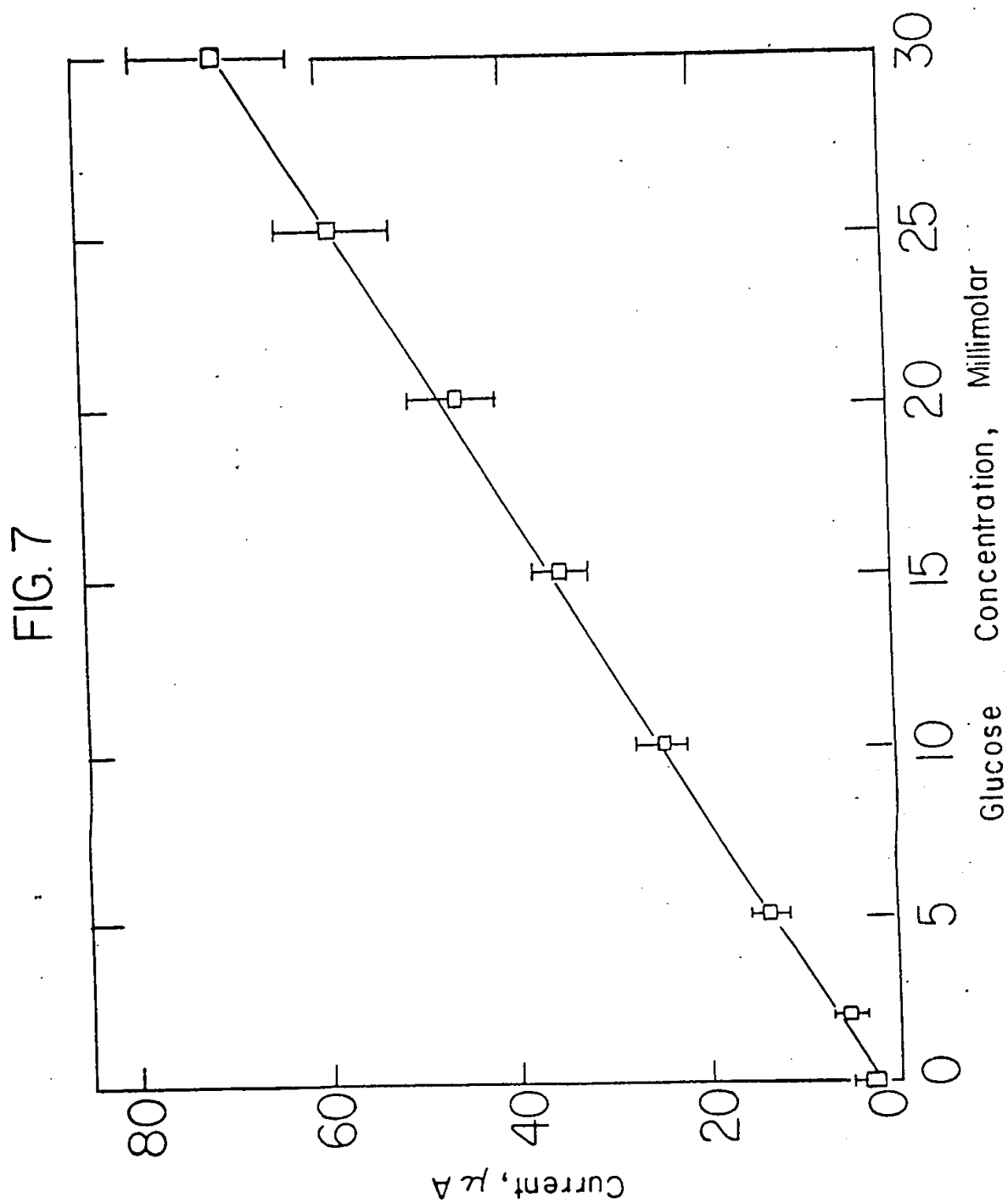


FIG. 3





SUBSTITUTE SHEET

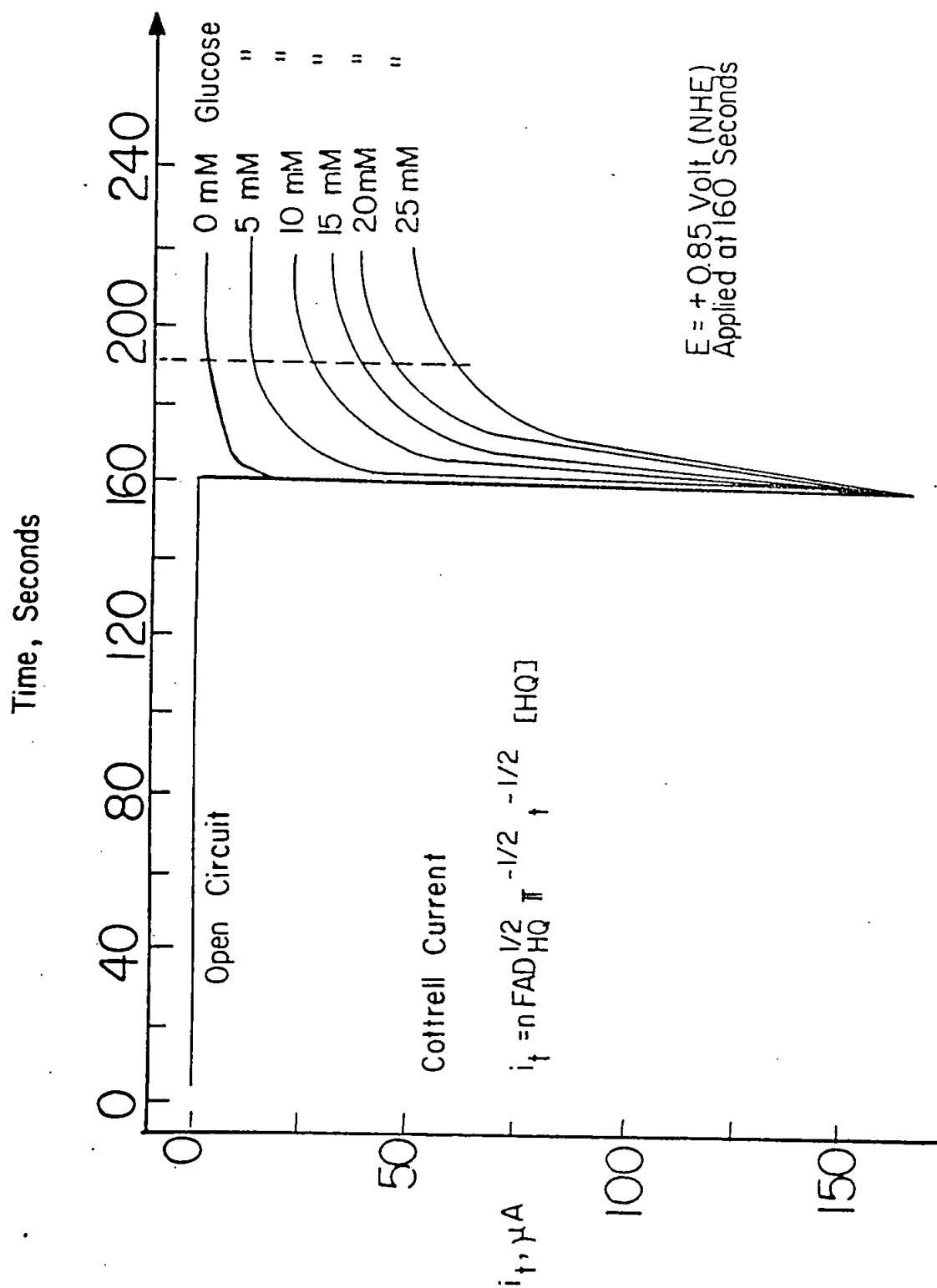


FIG.8

5/6

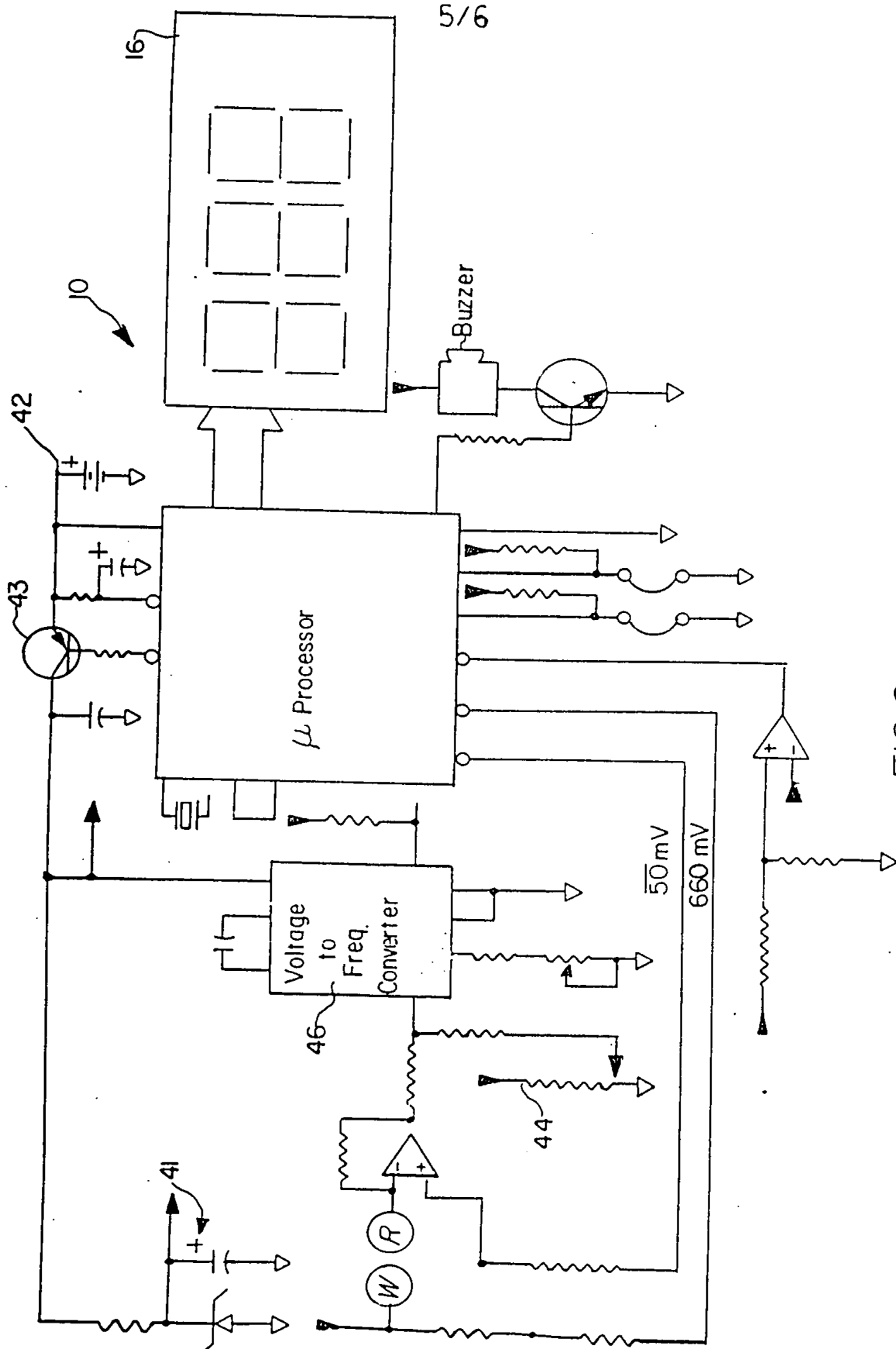
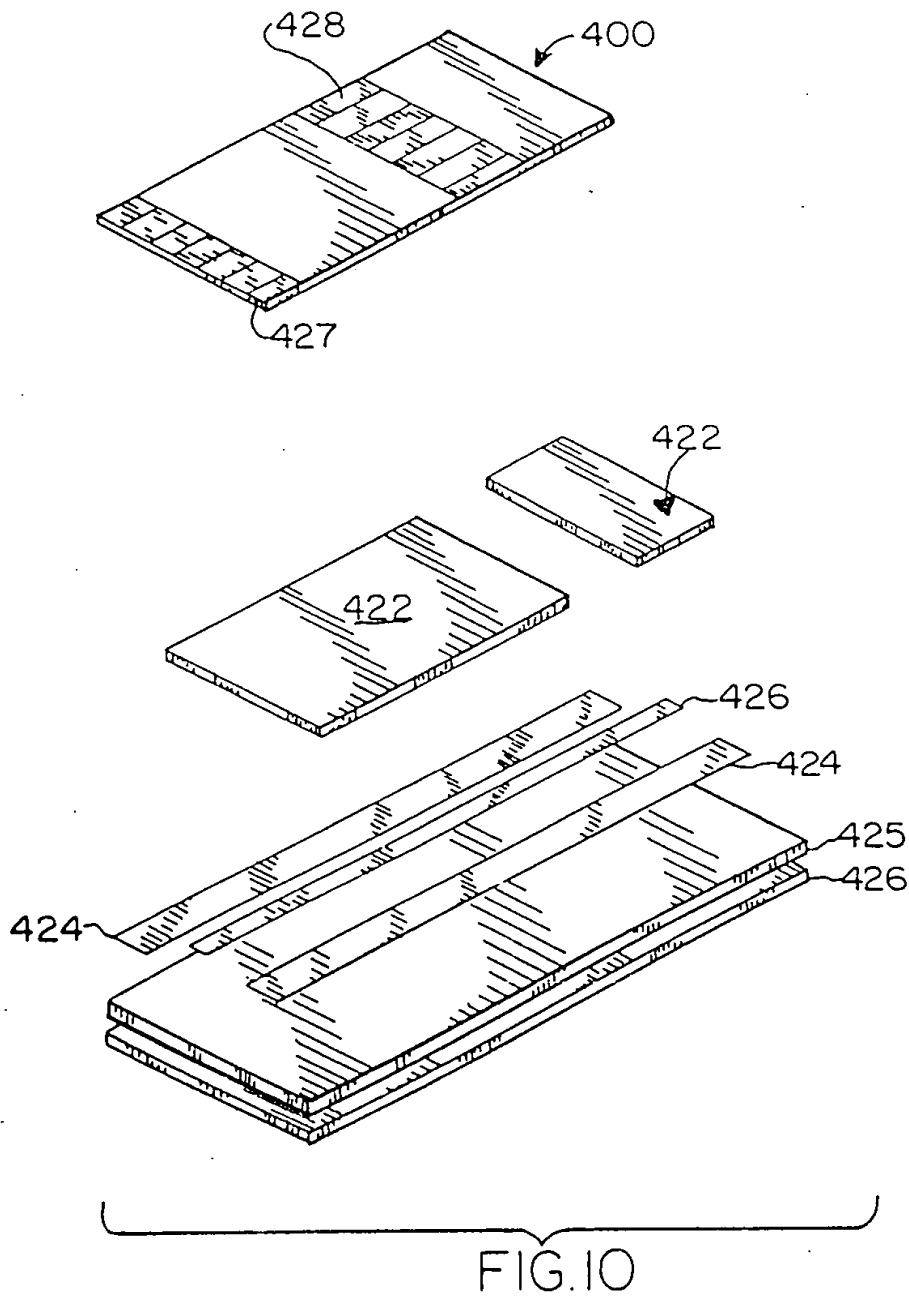


FIG. 9



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/01057

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all.)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12Q 1/00, 1/26; C12M 1/34; G01N 27/26

U.S. Cl. 435/4, 25, 291; 204/403

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System:

Classification Symbols

U.S.

435/4, 11, 14, 25, 288, 291, 817

204/403, 415

436/63, 150, 151, 817; 422/68, 73, 98

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, <sup>1</sup> with indication, where appropriate, of the relevant passages <sup>2</sup>	Relevant to Claim No. <sup>3</sup>
X	US, A, 4,005,002 (RACINE ET AL) 25 January 1977, See column 1, line 46 to column 2, line 25, column 3, lines 5 to 20, column 6, lines 15 to 33 and column 8, lines 16 to 17.	1-4
X	US, A, 3,838,033 (MINDT ET AL) 24 September 1974, See column 2, line 52 to column 3, line 19 and column 3, lines 46 to 75.	1-4
Y	US, A, 4,225,410 (PACE) 30 September 1980, See the entire document.	5-15
Y	US, A, 4,217,196 (HUCH) 12 August 1980, See column 3, line 35 to column 4, line 45.	5-15
Y	US, A, 3,925,183 (OSWIN ET AL) 09 December 1975, See column 2, lines 14 to 24 and column 5, line 28 to column 6, line 53.	5-11
Y	US, A, 4,169,779 (TATARIA ET AL) 02 October 1979, See column 3, line 18 to column 4, line 30.	5-11

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not  
considered to be of particular relevance

"E" earlier document but published on or after the international  
filing date

"L" document which may throw doubts on priority claim(s) or  
which is cited to establish the publication date of another  
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or  
other means

"P" document published prior to the international filing date but  
later than the priority date claimed

"T" later document published after the international filing date  
or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention

"X" document of particular relevance; the claimed invention  
cannot be considered novel or cannot be considered to  
involve an inventive step

"Y" document of particular relevance; the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

05 June 1989

Date of Mailing of the International Search Report

26 JUN 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

Randall E. Deck

## FURTHER INFORMATION, CONTINUED FROM THE SECOND SHEET

Y

US, A, 4,682,602 (PROHASKA) 28 July 1987  
 See column 2, lines 33 to 62 and column 3,  
 lines 24 to 59.

5-11

V ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_ because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remarks on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.